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Original Research Article

Clinical relevance of virulence genes in *Helicobacter pylori* isolates recovered from adult dyspeptic patients in Turkey

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ABSTRACT

Purpose: Bacterial virulence factors play a major role in the pathogenesis of *Helicobacter pylori* infection. The aims of this study were to evaluate virulence genes in *H. pylori* isolates and to compare the presence of these genes and associated clinical pathologies.

Methods: A total of 148 *H. pylori* isolates, recovered from adult dyspeptic patients, were used. The patients, from whom the isolates were obtained, were assigned to two groups by their endoscopic findings, which manifested as chronic gastritis or peptic ulcer. The presence of gastric atrophy and intestinal metaplasia was recorded for each patient, based on histopathological examination. Analyses of the virulence genes were performed by the polymerase chain reaction technique.

Results: The patients had a mean age of 47 ± 15 years and 86 (58%) of them were female. Based on endoscopic examination, 103 (69.6%) patients were diagnosed with chronic gastritis and 45 (30.4%) with peptic ulcer. Histopathological examination revealed intestinal metaplasia in 30 (20%) patients and gastric atrophy in 12 (8%) patients. The prevalence rates of *cagA, cagE, iceA1, iceA2,* and *babA2* were determined to be 87%, 74%, 58%, 26%, and 95%, respectively. The most prevalent *vacA* alleles were s1/s1a (82%/97%) and the least prevalent allele was s2 (20%). A new *vacA* genotype (s1as1bs1c) was detected, for the first time, in 18 (12%) isolates. No significant difference was found between the patient groups with chronic gastritis and peptic ulcer for the prevalences of the virulence genes ($p > 0.05$). Furthermore, intestinal metaplasia and gastric atrophy showed no significant correlation with the virulence genes ($p > 0.05$).

Conclusions: It is thoughted that *H. pylori* isolates with predominant *cagA, cagE, VacA* (s1, s1a), and *babA2* virulence genes are associated with gastroduodenal diseases. However, there is no correlation between gastric premalignant lesions and virulence genes.

1. Introduction

Helicobacter pylori, a gram-negative microaerophilic bacterium, is generally acquired at an early age by the oral route, and colonizes the gastric mucosa after being ingested. Unless treated, this gastric colonization persists for the lifetime of the host [1].

Following bacterial colonization, only 10–15% of infected individuals develop a symptomatic infection manifesting with signs such as gastritis, peptic ulcer, and gastric cancer, whereas a great majority remain asymptomatic [2]. Whether *H. pylori* colonization results in symptomatic infection is also defined by several factors pertaining to the bacterium, host, and environment [3]. The most important virulence factors of the bacterium include urease, the flagellar protein, cytotoxin-associated gene A (*cagA*), vacuolating cytotoxin gene A (*vacA*), epithelium A (*iceA*), and blood group antigen-binding adhesin gene (*babA*). These virulence genes have been reported to be associated with gastric diseases [3].

Cytotoxin-associated gene A (*cagA*), located at the end of the pathogenicity island (cag PAI), encodes a highly immunogenic and virulence-related protein. Reports indicate that *cagA*-positive *H. pylori* strains cause gastroduodenal disorders. The *CagE* gene, another member of the cag PAI, has also been reported to be associated with gastric disease [4].

The *vacA* gene, known to induce vacuolization in epithelial cells, is

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found in all *H. pylori* isolates. However, this gene varies in both structure and expression, which causes polymorphisms and different gastric pathologies [5]. Based on the nucleotide sequences of the signal (s) and mid (m) regions of its amino terminal, the *VacA* gene has been described to have allelic variants. The s region of the gene contains the s1 and s2 alleles, whilst the m region covers the m1 and m2 alleles. Different combinations of the s and m alleles create the s1m1, s1m2, s2m2, and s2m1 genotypes [6,7].

The *iceA* gene is also considered to be found in all *H. pylori* isolates. This gene has two alleles, referred to as *iceA1* and *iceA2* [8]. Gene *IceA1*, which regulates the contact of *H. pylori* with the gastric epithelium, is considered a potential marker of peptic ulcer [5].

To date, three different *bab* alleles (*babA1, babA2,* and *babB*) have been described, among which only the *babA2* gene encodes an adhesin that enables the adhesion of *H. pylori* to human Lewis b blood-group antigens. Thus, the most significant allele in terms of pathogenesis and clinical outcome is *babA2* [9].

This study was aimed at investigating the presence of the *cagA, cagE, vacA* (s1, s2, s1a, s1b, s1c, m1, m2), *iceA1, iceA2*, and *babA2* genes by molecular analysis in *H. pylori* isolates recovered from adult dyspeptic patients, and comparing these genes with the endoscopic and histopathological findings (intestinal metaplasia and gastric atrophy) of the patients.

2. Materials and methods

2.1. Patients

In a previous study conducted by the authors [10], a total of 148 patients were diagnosed to be positive for *H. pylori* by culture from 422 adult dyspeptic patients who underwent upper gastrointestinal endoscopy under sedation. This study enrolled these 148 patients, who were confirmed to be *H. pylori* positive by culture. The optimum sample size required for this study was determined by an online sample size calculator [11]. The patients were assigned to two groups according to their endoscopic findings as chronic gastritis or peptic ulcer in the current study. The diagnosis of chronic gastritis was confirmed histopathologically. Intestinal metaplasia and gastric atrophy were examined by an experienced pathologist.

2.2. Culture

The gastric biopsy specimens maintained in BHI broth (CM1135B, Thermo Fisher Scientific) were ground using a sterile glass rod and homogenized. Twenty µl of this material was inoculated onto Columbia blood agar base (CM0331B, Thermo Fisher Scientific) enriched with 10% defibrinated horse blood and Dent supplement (SR0147E, Thermo Fisher Scientific). The inoculated plates were incubated at 37 °C in a microaerobic atmosphere (Anaerocult C, Merck Millipore, Darmstadt, Germany) for 7–10 days. After the incubation period colonies grown were evaluated in terms of *H. pylori*.

2.3. Helicobacter pylori isolates

A total of 148 *H. pylori* isolates, recovered from the enrolled patients, were used for virulence gene analyses. The isolates were identified to be *H. pylori* by phenotypic tests (gram stain, oxidase, catalase, urease, motility, and colony morphology etc.) and then confirmed using molecular analysis (GlmM gene, F 5′-AAG CTT TTA GGG GTG TTA GGG GTT T-3′ and R 5′-AAG CTT ACT TTC TAA CAC TAA CGC-3′) [12]. The isolates were stored in brain-heart infusion (BHI) broth, containing 15% glycerol (CM1135B, Thermo Fisher Scientific, Waltham, MA, USA), at −84 °C.

2.4. DNA extraction

Frozen stocks of *H. pylori* isolates, stored at −84 °C, were cultured on Columbia blood agar base (Oxoid, Hampshire, UK), supplemented with 7% defibrinated horse blood, at 37 °C for 3–4 days under microaerobic conditions (Anaerocult C, Merck, Germany). Genomic DNA was extracted from each refreshed isolate by applying the single-cell lysis buffer (SCLB) procedure, as described by Olah et al. [13]. DNA extracts were stored at −20 °C until being used.

2.5. Virulence gene analyses

In the present study, the analyses of the *cagA, cagE, vacA* (s1, s2, s1a, s1b, s1c, m1, and m2 alleles), *iceA (iceA1* and *iceA2 alleles),* and *babA2* genes of the 148 *H. pylori* isolates were performed with the polymerase chain reaction (PCR) technique. The analysis of each gene was performed via singleplex PCR. The primers used in the study, as well as the gene names, expected band sizes and thermal cycle conditions are shown in Table 1. The amplified products were resolved by 1.5% agarose (Agarose NEEO ultra-quality, Carl Roth GmbH, Germany) gel electrophoresis and visualized under an UV transilluminator (G:BOX Chemi XRQ; Syngene, Cambridge, UK). Bands, the sizes of which are given in Table 1 for each gene, were considered as a positive result.

2.6. Standard strain

The *H. pylori* ATCC 700824 strain was used as a positive control in the molecular analyses.

2.7. Statistical analysis

Data were analyzed using the SPSS version 20.0 software (IBM Corporation, Armonk, NY, USA). The Kolmogorov-Smirnov test was used to assess the normal distribution of the continuous variables. Values were expressed as mean ± standard deviation for the normally distributed variables, and as count and percent for the categorical variables. In univariate analysis, the variables were compared using the chi-square test for categorical data. The Pearson correlation test was used to detect the possible correlation between the virulence genes. For all analyses, a p value of less than 0.05 was considered statistically significant.

2.8. Ethics approval

This study was approved by the Local Research Ethics Committee of the Medical Faculty of Erciyes University and designed in accordance with the 2013 Brazil version of the Helsinki Declaration. The Ethics Committee Reference Number is 2020/440.

3. Results

3.1. Clinical data

The mean age of the patients was 47 ± 15 years (range: 18–78). Of all patients, 86 (58%) were female. Based on endoscopic examination, 103 (69.6%) patients were diagnosed with chronic gastritis and 45 (30.4%) were diagnosed with peptic ulcer. Among the patients diagnosed with peptic ulcer, 41 (27.7%) had duodenal ulcer and four (2.7%) suffered from gastric ulcer. Histopathological examination demonstrated that 30 (20%) patients had intestinal metaplasia and 12 (8%) had gastric atrophy (Table 2).

3.2. Prevalence of the virulence genes

The prevalence rates of the *cagA, cagE, iceA1, iceA2,* and *babA2* virulence genes were determined to be 87%, 74%, 58%, 26%, and 95%, respectively. The prevalences of the s1, s1a, s1b, s1c, s2, m1, m2, s1s2, m1m2, s1m1, s1m2, s2m1, s2m2, s1as1bs1c, and s1s2m1m2 genotypes of the *vacA* gene were ascertained as 82%, 97%, 30%, 39%, 20%, 36%, 66%, 2.7%, 2.7%, 35%, 48%, 1.4%, 20%, 12%, and 1.4%, respectively.

Table 1
Primer sequence and PCR conditions used the current study.

Genes	Primer sequence (5′-3′)	Amplicon size (bp)	PCR conditions	References
vacA				
s1/s2	F-ATGGAAATACAACAAACACAC R-CTGCTTGAATGCGCCAAAC	259/286	94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min (35 cycles)	[6]
s1a	F-GTCAGCATCACACCGCAAC R-CTGCTTGAATGCGCCAAAC	190	94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min (35 cycles)	[6]
s1b	F-AGCGCCATACCGCAAGAG R-CTGCTTGAATGCGCCAAAC	187	94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min (35 cycles)	[6]
s1c	F-CTCTCGCTTTAGTGGGGYT R-CTGCTTGAATGCGCCAAAC	213	94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min (35 cycles)	[4]
m1/m2	F-CAATCTGTCCAATCAAGCGAG R-GCGTCAAAATAATTCCAAGG	567/642	94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min (35 cycles)	[7]
cagA	F-GATAACAGGCAAGCTTTTGAGG R-CTGCAAAAGATTGTTTGGCAGA	349	94 °C, 1 min; 57 °C, 1 min; 72 °C, 1 min (35 cycles)	[6]
cagE	F-TTGAAAACTTCAAGGATAGGATAGAGC R-GCCTAGCGTAATATCACCATTACCC	508	94 °C, 1 min; 53 °C, 45 s; 72 °C, 45 s (35 cycles)	[7]
iceA1	F-GTGTTTTTAACCAAAGTATC R-CTATAGCCATTATCTTTGCA	247	95 °C 1 min; 57 °C, 1 s; 72 °C, 1 min (35 cycles)	[7]
iceA2	F-GTTGGGTATATCACAATTTAT R-TTTCCCTATTTTCTAGTAGGT	229	95 8C 1 min; 57 8C, 1 s; 72 8C, 1 min (35 cycles)	[7]
babA2	F-CCAAACGAAACAAAAAGCGT R-GCTTGTGTAAAAGCCGTCGT	271	94 °C, 1 min; 45 °C, 1 min; 72 °C, 1 min (30 cycles)	[14]

Table 2
Demographic and clinical data of the patients (n: 148).

Characteristics	Results
Age, year (mean±SD)	47 ± 15 (range: 17–78)
Sex, n (%) (F/M)	86/62 (58/42)
Endoscopic findings, n (%)	
Chronic gastritis	103 (69.6)
Peptic ulcer	45 (30.4)
Duodenal ulcer	41 (27.7)
Gastric ulcer	4 (2.7)
Histopathological findings, n (%)	
Intestinal metaplasia	30 (20)
Gastric atrophy	12 (8)

SD: Standard deviation, F: Female, M: Male.

The most prevalent allele of the *vacA* gene was s1a (97%), and the least prevalent allele was s2 (20%). Among the allele combinations of the *vacA* gene, the most prevalent was s1m2 (48%) and the least prevalent were s2m1 (1.4%) and s1s2m1m2 (1.4%) (Table 3).

Table 3
Prevalence of the virulence genes and distribution of these genes according to endoscopic findings.

Genes	Chronic gastritis (n: 103)	Peptic ulcer (n: 45)	*p*	Total (n: 148)
cagA, n (%)	91 (88)	38 (84)	0.51	129 (87)
cagE, n (%)	75 (73)	35 (78)	0.52	110 (74)
vacA, n (%)				
s1	83 (81)	39 (87)	0.37	121 (82)
s1a	99 (96)	45 (100)	0.82	144 (97)
s1b	27 (26)	17 (38)	0.15	44 (30)
s1c	37 (36)	21 (47)	0.21	58 (39)
s2	22 (21)	8 (18)	0.61	30 (20)
m1	37 (36)	15 (33)	0.76	53 (36)
m2	67 (65)	31 (69)	0.65	98 (66)
s1s2	2 (2)	2 (4)	0.54	4 (2.7)
m1m2	3 (3)	1 (2)	0.81	4 (2.7)
s1m1	37 (36)	15 (33)	0.76	52 (35)
s1m2	47 (46)	25 (56)	0.26	71 (48)
s2m1	1 (1)	1 (2)	0.54	2 (1.4)
s2m2	22 (21)	8 (18)	0.61	30 (20)
s1as1bs1c	10 (10)	8 (18)	0.16	18 (12)
s1s2m1m2	1 (1)	1 (2)	0.54	2 (1.4)
iceA1, n (%)	57 (55)	30 (67)	0.19	86 (58)
iceA2, n (%)	27 (26)	11 (24)	0.82	38 (26)
babA2, n (%)	97 (94)	44 (98)	0.67	141 (95)

3.3. Correlation between the virulence genes and endoscopic/histopathological findings

No statistically significant difference was detected between the chronic gastritis group and the peptic ulcer group for the prevalence rates of the *H. pylori* virulence genes ($p > 0.05$) (Table 3). Moreover, no significant correlation existed between intestinal metaplasia/gastric atrophy and the *H. pylori* virulence genes ($p > 0.05$) (Table 4).

4. Discussion

The results of previous studies regarding the prevalence of *H. pylori* virulence genes, and the correlation between these genes and clinical findings show significant variances, such that even results from various regions of a country differ [15–18].

The prevalences of *cagA*, *cagE, iceA1, iceA2, and babA2* have been reported to range between 48% and 98%, 41%–88%, 21%–96%, 16%–79%, and 23%–95%, respectively, across the world [15–29]. The predominant *vacA* allele has been reported as s1 (56%–100%) in most studies conducted in different regions of the world [15,20–23,25,28,30],

Table 4
Prevalence of the virulence genes according to histopathological findings.

Genes	IM (+) (n: 30)	IM (−) (n: 118)	*p*	GA (+) (n: 12)	GA (−) (n: 136)	*p*
cagA, n (%)	24 (80)	105 (89)	0.2	10 (83)	119 (88)	0.7
cagE, n (%)	24 (80)	86 (78)	0.4	9 (75)	101 (74)	0.9
vacA, n (%)						
s1	26 (87)	96 (81)	0.5	10 (83)	112 (82)	0.9
s1a	29 (97)	115 (98)	0.8	12 (100)	132 (97)	0.6
s1b	10 (33)	34 (29)	0.6	3 (25)	41 (30)	0.7
s1c	10 (33)	34 (29)	0.8	5 (42)	53 (39)	0.8
s2	4 (13)	26 (22)	0.3	2 (17)	28 (21)	0.7
m1	10 (33)	42 (36)	0.8	5 (42)	47 (35)	0.6
m2	19 (63)	79 (67)	0.7	6 (50)	92 (67)	0.2
s1s2	0 (0)	4 (3)	0.3	0 (0)	4 (3)	0.5
m1m2	0 (0)	4 (3)	0.3	0 (0)	4 (3)	0.5
s1m1	10 (33)	42 (36)	0.8	5 (42)	47 (35)	0.6
s1m2	15 (50)	57 (48)	0.9	4 (33)	68 (50)	0.3
s2m1	0 (0)	2 (1.7)	0.5	0 (0)	2 (1.5)	0.7
s2m2	4 (13)	26 (22)	0.3	2 (17)	28 (21)	0.7
s1as1bs1c	2 (7)	16 (14)	0.3	2 (17)	16 (12)	0.6
s1s2m1m2	0 (0)	2 (1.7)	0.5	0 (0)	2 (1.5)	0.7
iceA1, n (%)	18 (60)	69 (59)	0.9	8 (67)	79 (58)	0.6
iceA2, n (%)	7 (23)	31 (26)	0.7	2 (17)	36 (27)	0.5
babA2, n (%)	29 (97)	112 (95)	0.7	11 (92)	130 (96)	0.6

IM: Intestinal metaplasia, GA: Gastric atrophy, +: Positive, -: Negative.

and as m2 (68%) in two studies [24,29]. Our results (Table 3) are in agreement with the majority of the studies mentioned above.

We also showed that, among the allele combinations of the *VacA* gene, the most prevalent was s1m2 (48%) and the least prevalent was s2m1 (1.4%) (Table 3). In view of the very low prevalence (0.6%–5%) reported for the s2m1 allele combination of the *vacA* gene in some studies [21,28] and the determination of the inexistence of such an allele combination in some other studies [18,24,29,30], the existence of this allele is still controversial.

An interesting result obtained in the present study was the detection of the *vacA* s1s2, m1m2, and s1s2m1m2 allele combinations in four (2.7%), four (2.7%), and two (1.4%) isolates, respectively (Table 3). Percentages of isolates possessing the s1s2, m1m2, and s1s2m1m2 allele combinations have been reported as 16%, 36%, and 4%, respectively, by Tanih et al. [30] and as 8%, 8%, and 3%, respectively, by Gatti et al. [25]. Miernyk et al. [20] reported the percentages of isolates carrying the s1s2 and m1m2 allele combinations as 5% and 3%, respectively. Tanih et al. [30] demonstrated that all patients with the *vacA* s1s2m1m2 allele combination had peptic ulcer. Nevertheless, the present study showed that no significant correlation existed between these allele combinations and the clinical findings of the patients. This could be due to these allele combinations having been detected in only very few of the patients. We also detected the *vacA* s1as1bs1c allele combination in 18 isolates (12%). To our knowledge, this allele combination has not been reported in the literature before. In this study, in which we report the *vacA* s1as1bs1c allele for the first time, we found no significant correlation between the two patient groups for this particular allele combination.

As indicated above, the prevalence rates of virulence genes vary in different regions of the world. These differences could be related to different methods and primers having been used for the detection of the genes, as well as geographical and ethnic varieties, and differences in the number and clinical findings of the patients from whom the isolates were obtained.

While most studies have shown that the *cagA, cagE,* and *vacA* virulence genes are associated with gastroduodenal diseases [15,18–20, 22–25,27], some other have suggested no existing correlation [16,26, 28–30]. On the other hand, no correlation has been reported for the *iceA* gene either in several studies [15,19,24,29,30]. Among the studies reviewed for the correlation of the *babA2* gene allele with gastroduodenal diseases, while one showed that the prevalence of the *babaA2* gene allele was correlated with chronic gastritis [25], the others reported no such correlation [15,19,24,29].

It is clear that literature data available on the correlation of *H. pylori* virulence genes with gastroduodenal diseases is conflicting. Although no statistically significant correlation was found between the chronic gastritis group and the peptic ulcer group for the positivity rates of the virulence genes in the present study, it is considered that a correlation could exist between the predominant virulence genes (*cagA, cagE, babA2,* and *vacA* (s1/s1a alleles)) and gastroduodenal diseases (Table 3). A similar speculation has been made earlier [19].

Only very few studies have been carried out on the correlation of the virulence genes with gastric premalignant lesions such as intestinal metaplasia, gastric atrophy, and dysplasia. To date, no correlation has been able to be demonstrated between most virulence genes and gastric premalignant lesions [22,26]. Similar to these literature reports, our study revealed no correlation between intestinal metaplasia/gastric atrophy and the virulence genes (Table 4).

The most important limitation of the current study is that since this study is monocentric, the results obtained may not be representative of the whole of Turkey. Another limitation of the study is that there were no patients with gastric malignancies among the patients included in the study. The most notable strength of the study is that this study is the most comprehensive research ever conducted in Turkey, considering the number of *H. pylori* isolates used and the virulence genes analyzed. Another strength of the study is that the *vacA* s1as1bs1c genotype was detected in 18 (12%) *H. pylori* isolates for the first time in this study.

5. Conclusion

The *cagA, cagE, babA2,* and *vacA* (s1/s1a alleles) genes were determined to be predominant. As all the patients enrolled in the present study were diagnosed with chronic gastritis or peptic ulcer, it is considered that a correlation could exist between these predominant virulence genes and gastroduodenal diseases. However, no correlation between the gastric premalignant lesions (intestinal metaplasia and gastric atrophy) and the virulence genes were detected in our study. The multicenter studies to be conducted on large heterogeneous patient groups are needed to reveal the exact relationship between the predominant virulence genes and gastroduodenal diseases.

CRediT authorship contribution statement

Mustafa Akar: Conceptualization, Methodology, Formal analysis, Software, Data curation, Writing – review & editing. **Tuba Kayman:** Investigation, Data curation, Writing – review & editing. **Seçil Abay:** Investigation, Resources, Writing – review & editing. **Tevfik Solakoğlu:** Investigation, Writing – review & editing. **Emre Karakaya:** Investigation, Writing – review & editing, Resources. **Fuat Aydin:** Supervision, Writing – review & editing, Resources.

Declaration of competing interest

The manuscript has not been published previously elsewhere. There is no specific funding has been received to report this submission. All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version. All authors are in agreement with the content of the manuscript. The authors have no conflict of interest to disclose.

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